JVI Journals.ASM.org

Influenza A Virus Polymerase Is a Site for Adaptive Changes during Experimental Evolution in Bat Cells

Daniel S. Poole,[a] Shuǐqìng Yú,[b] Yíngyún Caì,[b] Jorge M. Dinis,[c] Marcel A. Müller,[d] Ingo Jordan,[e] Thomas C. Friedrich,[c,f] Jens H. Kuhn,[b] Andrew Mehle[a]

Department of Medical Microbiology and Immunology, University of Wisconsin—Madison, Madison, Wisconsin, USA[a]; NIH/NIAID Integrated Research Facility at Fort Detrick, Frederick, Maryland, USA[b]; Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin—Madison, Madison, Wisconsin, USA[c]; Institute of Virology, University of Bonn Medical Centre, Bonn, Germany[d]; ProBioGen AG, Berlin, Germany[e]; Wisconsin National Primate Research Center, Madison, Wisconsin, USA[f]

ABSTRACT

The recent identification of highly divergent influenza A viruses in bats revealed a new, geographically dispersed viral reservoir. To investigate the molecular mechanisms of host-restricted viral tropism and the potential for transmission of viruses between humans and bats, we exposed a panel of cell lines from bats of diverse species to a prototypical human-origin influenza A virus. All of the tested bat cell lines were susceptible to influenza A virus infection. Experimental evolution of human and avian-like viruses in bat cells resulted in efficient replication and created highly cytopathic variants. Deep sequencing of adapted human influenza A virus revealed a mutation in the PA polymerase subunit not previously described, M285K. Recombinant virus with the PA M285K mutation completely phenocopied the adapted virus. Adaptation of an avian virus-like virus resulted in the canonical PB2 E627K mutation that is required for efficient replication in other mammals. None of the adaptive mutations occurred in the gene for viral hemagglutinin, a gene that frequently acquires changes to recognize host-specific variations in sialic acid receptors. We showed that human influenza A virus uses canonical sialic acid receptors to infect bat cells, even though bat influenza A viruses do not appear to use these receptors for virus entry. Our results demonstrate that bats are unique hosts that select for both a novel mutation and a well-known adaptive mutation in the viral polymerase to support replication.

IMPORTANCE

Bats constitute well-known reservoirs for viruses that may be transferred into human populations, sometimes with fatal consequences. Influenza A viruses have recently been identified in bats, dramatically expanding the known host range of this virus. Here we investigated the replication of human influenza A virus in bat cell lines and the barriers that the virus faces in this new host. Human influenza A and B viruses infected cells from geographically and evolutionarily diverse New and Old World bats. Viruses mutated during infections in bat cells, resulting in increased replication and cytopathic effects. These mutations were mapped to the viral polymerase and shown to be solely responsible for adaptation to bat cells. Our data suggest that replication of human influenza A viruses in a nonnative host drives the evolution of new variants and may be an important source of genetic diversity.

Influenza A virus (FLUAV) infects a broad range of mammalian and avian hosts (1, 2). Transmission of FLUAV from natural reservoirs (migratory water fowl) to domestic hosts (poultry and pigs) and ultimately into the human population routinely involves the mutation of viral genes, the exchange of genes between viruses during the process of reassortment, or both (3–5). Pigs, susceptible to both avian and human influenza A viruses, function as mixing vessels for these evolutionary processes that have resulted in viruses causing human pandemics. A defining feature of the porcine mixing vessel is the ability to be infected by both human and animal influenza A viruses. Therefore, birds and pigs are central to any FLUAV surveillance efforts. However, results from several recent studies have revealed a reservoir much broader and a pattern of cross-species transmission more complex than those that were previously appreciated: identification of nonhuman primates naturally infected by viruses closely related to human FLUAV (6), natural infection and disease in domestic cats caused by highly pathogenic avian H5N1 viruses (7, 8), transmission of H3N8 equine FLUAV into dogs that evolved into a lineage of canine influenza A virus that causes severe respiratory disease (9), a distinct lineage of avian influenza virus in Antarctic penguins (10), a potentially new genus for orthomyxoviruses in pigs and cattle distantly related to influenza C virus (11), and identification of harbor seals and farmed guinea pigs infected by both FLUAV and influenza B virus (FLUBV) (12–14) (previously, FLUBV was thought to exclusively infect humans [15]). Amplifying these reports, the recent detection of highly divergent FLUAVs in bats revealed a potentially vast new host range, as bats (order Chiroptera) comprise ≈20% of all classified mammals (16–19).

Bats are natural reservoirs for a wide variety of viruses (20, 21), including many important zoonotic viruses that can cause severe disease, such as the filoviruses Marburg and Ravn (22, 23), the paramyxoviruses Hendra and Nipah (24, 25), and coronaviruses

Received 26 June 2014 Accepted 12 August 2014
Published ahead of print 20 August 2014
Editor: T. S. Dermody
Address correspondence to Andrew Mehle, amehle@wisc.edu.

doi:10.1128/JVI.01857-14

(CoVs) closely related to Middle Eastern respiratory syndrome (MERS)-CoV and severe acute respiratory syndrome (SARS)-CoV (26–30). Very recently, bats have been shown to harbor hepaciviruses, hepadnaviruses, paramyxoviruses, and pegiviruses, which may be the ancestors of the hepatitis C, hepatitis B, mumps, and GB viruses currently circulating in humans, respectively (31–33). These findings suggest that bats harbor many viruses known to spill over into humans, and in some cases this spillover may involve complete host switching between bats and other mammals (34).

Two new lineages of FLUAV, H17N10 and H18N11, have recently been identified in New World bats (16, 17). H17N10 and H18N11 are the first known bat FLUAVs and were detected in microbats that belong to separate genera and that were sampled over 3,000 km apart. Phylogenetic analysis revealed these two viruses to be highly divergent from other extant FLUAVs, indicating an ancient origin coupled with long-term transmission and evolution in bats. Although isolates of these viruses have not yet been reported, for public health it is important to determine the potential of bats to serve as reservoirs or to be infected by human or avian viruses and act as mixing vessels for FLUAV variants capable of (re)entering the human population.

To infect a new host, FLUAV must overcome significant barriers to cross-species transmission and replication. Whereas proteins encoded on all eight genomic RNA segments of FLUAV have been implicated in this process, the dominant contributors are the segments encoding the cell surface hemagglutinin (HA) and the RNA-dependent RNA polymerase (RdRP) complex (1, 35, 36). HA mediates the attachment and entry of virions into cells by binding sialic acid moieties on the host cell surface. Species-specific variations in the sialic acid structures create a major barrier to cross-species transmission that is overcome by adaptive HA mutations (37–39). The viral neuraminidase (NA) protein also binds sialic acids, cleaving the glycan to facilitate release and prevent the self-aggregation of virions. The specificity of NA evolves along with HA to recognize species-specific sialic acid variants (40, 41). Importantly, biochemical analyses indicate that bat FLUAV-derived H17 and H18 proteins do not bind sialic acids typically used by known FLUAV variants or any of more than 600 glycans tested (17, 42). Moreover, the bat FLUAV NA-like proteins N10 and N11 lack sialidase activity (17, 43, 44). The structures of bat HA- and NA-like proteins reveal highly divergent receptor binding sites, likely contributing to their altered target specificity (17, 42–45).

The FLUAV RdRP, composed of subunits PB1, PB2, and PA, associates with genomic RNA and the viral nucleoprotein (NP) to form ribonucleoprotein (RNP) complexes that mediate transcription and replication (46). Unlike the replication of most other RNA viruses, the replication of FLUAV occurs in the nucleus of the host cell. RdRPs derived from avian FLUAV generally function poorly in human cells (47, 48), with data suggesting the presence of a potent restriction factor present in human cells that selectively impairs polymerase function (49). Restricted RdRPs cannot support efficient virus replication due to inhibition of RNP formation and subsequent genome transcription and replication, although the enzymatic activity of the complex appears to be largely unaffected (49–52). A single mutation in the PB2 subunit, conversion of the avian signature glutamic acid residue at position 627 to the human signature lysine residue (E627K), is sufficient to overcome FLUAV restriction in human cells (47, 49, 50, 53, 54). Other FLUAV restriction escape mechanisms include second-site suppressor mutations and alterations in the nuclear import sequence of the PB2 subunit, as well as reassortment involving the PA subunit (36, 55–59). Reassortment of genes in the viral replication machinery (i.e., PB1, PB2, PA, and NP) contributed to the emergence of pandemic FLUAV strains in 1957, 1968, and 2009 (60–62). The restrictive FLUAV barriers present in bats, if any, and the relationship of these barriers to viral host range and the potential for bats to be reservoirs are unknown.

A critical question is whether FLUAV can move back and forth between humans and bats and which barriers are faced in a heterotypic host. Transmission of viruses from bats into humans has precedents and can be fatal in the case of diseases caused by Marburg virus, Hendra virus, Nipah virus, and SARS-CoV (22–27, 29). Similarly, FLUAV has crossed into humans and then back into mixing vessels. The 2009 H1N1 human influenza pandemic originally emerged from infected pigs and was subsequently transferred back to naive pigs by infected humans (60, 62, 63). Results presented here show that cells from a large number of bat species were susceptible to human FLUAV and that no intrinsic blockade existed at the cellular level to prevent infection by human viruses in bats. Rather, our results indicate that FLUAV quickly adapted in bat cells to replicate to high titers in a new potential host. These findings highlight the importance of defining the full scope of mixing vessels and viral reservoirs that contribute to human disease.

MATERIALS AND METHODS

Cells. MDCK, MDBK, HEK293T, DF-1, and PK(15) cells (ATCC, Manassas, VA) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). HA-MDCK cells were grown in DMEM supplemented with 10% FBS and 0.2 mg/ml hygromycin B (64). LMH cells (a gift from D. Loeb) and Calu-3 cells (a gift from R. Baric) were grown in DMEM–Ham's F-12 medium (DMEM/F-12) supplemented with 5% and 20% FBS, respectively. The bat cell lines used in this study are described in Table 1. The R05T, R06E, and HypNi/1.1 cell lines were grown in DMEM/F-12 supplemented with 10% FBS, Tb 1 Lu cells were grown in DMEM or minimal essential medium alpha (Mediatech, Inc., Manassas, VA) supplemented with 10% FBS, and all other bat cell lines were maintained in DMEM supplemented with 10% FBS. All cells were grown at 37°C in 5% CO_2.

Plasmids. Plasmids carrying FLUAV RdRP proteins and NP were derived from FLUAV strains A/WSN/33 (H1N1; here abbreviated WSN), A/green-winged teal/OH/175/86 (H2N1; S009), A/Brevig Mission/18 (H1N1; 1918), A/Udorn/72 (H3N2; Udorn), A/New York/312/2001 (H1N1; NY312), and A/Utah/1/2009 (H1N1; UT1) and have been described previously (49, 55, 65). A/little yellow-shouldered bat/Guatemala/164/2009 (H17N10; Guat164) genes were synthesized by GeneArt (Life Technologies, Grand Island, NY). The PA gene was cloned to express a PA with a FLAG epitope tag at the C terminus. pCDNA6.2-HA was used to express HA cloned from the WSN strain. The vNA-Luc reporter plasmid was used to express a minus-sense luciferase (Luc) gene flanked by the untranslated regions derived from WSN viral NA (vNA) using a polymerase I (*polI*) promoter and terminator (49, 66). The virus rescue plasmid pTM-ΔRNP (55) was derived from pTM-All (a gift from Y. Kawaoka [67]), used to express HA, NA, M, and NS viral RNAs (vRNAs) from the WSN strain. pBD-PB1, -PB2, -PA, -NP, -NA, -M, and -NS contained bidirectional *polI* and *polII* cassettes to express both vRNA and mRNA (49, 68). pPOLI-HA(45)GFP (69) was a kind gift from P. Palese (64). Mutant constructs were created by PCR-based strategies and confirmed by sequencing.

Viruses. Wild-type (WT) and reassorted WSN viruses were rescued by cotransfecting a coculture of HEK293T and MDCK cells with pTM-ΔRNP and the pBD plasmids using the TransIT-2020 transfection reagent

TABLE 1 Origin of bat cell lines used in this study

Cell line	Reference or source	Origin bat	Origin tissue	Taxonomy of origin bat	Diet of origin bat
EidNi/41.3	74	African straw-colored fruit bat (*Eidolon helvum*)	Kidney	Old classification, Megachiroptera: Pteropodidae; new classification, Yinpterochiroptera: Pteropodidae	Fruit
EpoNi/22.1	99	Büttikofer's epauletted fruit bat (*Epomops buettikofei*)	Adult kidney	Old classification, Megachiroptera: Pteropodidae; new classification, Yinpterochiroptera: Pteropodidae	Fruit
HypLu/45.1	99	Hammer-headed fruit bat (*Hypsignathus monstrosus*)	Fetal lung	Old classification, Megachiroptera: Pteropodidae; new classification, Yinpterochiroptera: Pteropodidae	Fruit
HypNi/1.1	99	Hammer-headed fruit bat (*Hypsignathus monstrosus*)	Fetal kidney	Old classification, Megachiroptera: Pteropodidae; new classification, Yinpterochiroptera: Pteropodidae	Fruit
MyDauLu/47.1	99	Daubenton's myotis (*Myotis daubentonii*)	Adult lung	Old classification, Microchiroptera: Vespertilionidae; new classification, Yangochiroptera: Vespertilionidae	Insects
PeSu B5L	100	Eastern pipistrelle (*Pipistrellus* [*Perimyotis*] *subflavus*)	Adult lung	Old classification, Microchiroptera: Vespertilionidae; new classification, Yangochiroptera: Vespertilionidae	Insects
R05T	101	Egyptian rousette (*Rousettus* [*Rousettus*] *aegyptiacus*)	Embryo head	Old classification, Megachiroptera: Pteropodidae; new classification, Yinpterochiroptera: Pteropodidae	Fruit
R06E	101	Egyptian rousette (*Rousettus* [*Rousettus*] *aegyptiacus*)	Embryo body	Old classification, Megachiroptera: Pteropodidae; new classification, Yinpterochiroptera: Pteropodidae	Fruit
RoNi/7.1	99	Egyptian rousette (*Rousettus* [*Rousettus*] *aegyptiacus*)	Adult kidney	Old classification, Megachiroptera: Pteropodidae; new classification, Yinpterochiroptera: Pteropodidae	Fruit
RoNi/7.2	RoNi/7 subclone (76)	Egyptian rousette (*Rousettus* [*Rousettus*] *aegyptiacus*)	Adult kidney	Old classification, Megachiroptera: Pteropodidae; new classification, Yinpterochiroptera: Pteropodidae	Fruit
Tb 1 Lu	ATCC (CCL-88)	Brazilian free-tailed bat (*Tadarida brasiliensis*)	Lung	Old classification, Microchiroptera: Molossidae; new classification, Yangochiroptera: Molossidae	Insects

(Mirus Bio, Madison, WI). Media were replaced 24 h later with virus growth medium [VGM; DMEM, 0.3% bovine serum albumin, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 0.5 μg/ml of L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) trypsin]. Viruses were harvested after an additional 24 to 72 h of incubation and amplified in MDBK cells to obtain low-passage-number parental stocks. The titers of all viruses were determined in MDCK cells overlaid with medium containing 1.2% Avicel cellulose (RC-581; FMC BioPolymer, Philadelphia, PA) (70). Mutant viruses were confirmed by sequencing of cDNA derived from the viral genomes. Single-cycle green fluorescent protein (GFP)-

expressing WSN virus was rescued in HEK293T cells cotransfected with pCDNA6.2-HA, pPOLI-HA(45)GFP (69), and the seven pBD plasmids. Virus was amplified, and the titer was determined on HA-MDCK cells. Vesicular stomatitis Indiana virus (VSV) carrying GFP (71) and FLUAV carrying VSV G glycoprotein (VSV-G) and GFP (FVG-G) (72) have been described previously.

Infection of bat cell lines with WSN. Bat cell lines were seeded in poly-D-lysine-coated 96-well plates (Greiner Bio-One, Monroe, NC) at 3×10^4 cells/well. One day later, the medium was removed and the cells were washed once with DMEM without FBS (0% DMEM). The cells were then infected with the single-cycle GFP-expressing WSN at a multiplicity of infection (MOI) of 0.2, 0.5, 2, or 5. After 1 h of incubation at 37°C, the viral inocula were removed and the cells were washed once with 0% DMEM and then supplemented with DMEM containing 2% FBS (2% DMEM). At 24 h postinoculation, one set of cells was fixed with 4% paraformaldehyde. GFP-positive cells were detected using high-content imaging (Operetta; PerkinElmer, Waltham, MA). The other set of cells was trypsinized and transferred to round-bottom 96-well plates. The percentage of GFP-positive cells was measured by fluorescence-activated cell sorting.

For viral entry studies, Brazilian free-tailed bat Tb 1 Lu cells were treated with receptor-destroying enzyme (RDE) or mock treated for 1 h prior to infection. RDE contained in *Vibrio cholerae* filtrate (Sigma-Aldrich, St. Louis, MO) was added at 5 μl/ml to medium supplemented with 100 μg/ml $CaCl_2$. Tb 1 Lu cells were infected with single-cycle GFP-expressing WSN reporter virus, a VSV-G-pseudotyped influenza virus expressing GFP (FVG-G), or VSV expressing GFP and imaged at 20 h postinoculation.

RdRP activity assays. HEK293T, LMH, or DF-1 cells were transfected in triplicate with plasmids carrying the PA-FLAG, PB1, PB2, and NP genes and the vNA-Luc reporter plasmid. Reassorted polymerases were created by exchanging subunits between human, bat, and avian isolates. The cells were lysed 24 to 48 h later in cell culture lysis reagent, and luciferase activity was measured using the luciferase assay system (Promega, Madison, WI). PA expression was confirmed by Western blotting.

Multicycle replication assays. Cells were infected in triplicate at the MOI indicated below in VGM or DMEM/F-12 VGM with 0.25 μg/ml TPCK trypsin, as appropriate. Infections in LMH, PK(15), and Tb 1 Lu cells were performed at 37°C. Infections in Calu-3 cells were performed at 33°C. The viral titers of aliquots taken throughout the infection were determined by plaque assay in MDCK cells.

Experimental evolution. Tb 1 Lu cells were initially infected with FLUAV (WT or reassortant WSN virus carrying the avian S009 RNP [WSN-S009 RNP]) at an MOI of 0.1. At 48 to 72 h postinoculation, virus was passaged blindly onto fresh Tb 1 Lu cells using a 1:10 to 1:100 dilution of viral supernatant for nine serial passages. Titers for passages 2 to 9 were determined by plaque assay on MDCK cells after all of the passaging had been completed and indicated that passages were performed at an MOI of ≈0.1. A final high-titer stock was created at the 10th passage.

Metabolic labeling. Tb 1 Lu cells were infected with FLUAV at an MOI of 1 for 9.5 h, transferred to medium lacking cysteine and methionine for 1 h, and subsequently labeled for 1.25 h with [^{35}S]Cys/Met EXPRE^{35}S^{35}S protein labeling mix (PerkinElmer). Whole-cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the gels were dried, and proteins were detected by phosphorimaging.

Deep sequencing. Viral cDNA was generated from viral stocks and samples from passages 6 and 9 as described previously (73). Individual FLUAV gene segments from each sample were gel purified, quantitated, and pooled in equimolar amounts into a single reaction mixture. Samples were fragmented, indexed with a Nextera DNA sample preparation kit, and sequenced on a MiSeq platform (Illumina, San Diego, CA). Sequence data were analyzed with the CLC Genomic Workbench (v.4.5.1; CLC Bio, Aarhus, Denmark). Sequence reads were deconvoluted and trimmed using a quality limit threshold of 0.001. Only reads greater than 100 bp in length were retained. Reads were mapped to full-length reference sequences from the cDNA clones used during the initial rescue of the parental virus (WSN) or WSN carrying the S009 RNP. SNPs were called at mapping sites with a greater than 100× coverage, a central base Phred quality score of Q30 or greater, and a frequency at or above 1%. High-frequency mutations found in the deep sequencing of adapted viruses were introduced into recombinant viruses that were used to infect Tb 1 Lu cells.

Statistical analysis. Experiments were performed in triplicate or more. Data are presented as the mean ± standard deviation (SD). Results from polymerase activity assays were normalized to those for the homotypic polymerase for each isolate, and error was propagated throughout the normalization. Statistical significance was determined by an unpaired two-tailed Student's *t* test.

RESULTS

Replication of human influenza A virus in bat cells. The discovery of unique FLUAV variants in New World microbats raised the possibility that bats may influence the dissemination of FLUAV to other mammals by acting as direct reservoirs or mixing vessels for the generation of reassortants (16, 17). To determine if bats could function as mixing vessels, we first assessed whether diverse bat cell lines are susceptible to infection with the prototypical mammalian H1N1 FLUAV A/WSN/33 (WSN), a laboratory-adapted human isolate. Bat cell lines were exposed to a single-cycle green fluorescent protein (GFP) reporter (GFP-WSN) virus at increasing multiplicities of infection (MOIs) (Fig. 1) (64). Cell lines were derived from African (EidNi/41.3, EpoNi/22.1, HypLu/45.1, HypNi/1.1, R05T, R06E, RoNi/7.1, RoNi/7.2), American (B5L, Tb 1 Lu), and Eurasian (MyDauLu/47.1) bats (Table 1). The cell lines represented multiple tissue types from embryonic and adult bats. At least one of these cell lines (EidNi/41.3) is known to express type I interferon (74). All cell lines tested were susceptible to infection with GFP-WSN virus to some degree. Similar to control infections in dog kidney (MDCK) cells, the percentage of infected cells mostly increased with increasing MOI. The apparent decrease in GFP-positive cells detected by microscopy for infections in R06E cells resulted from the high degree of cytopathic effect, cell death, and detachment from the growth surface, even though fluorescence-activated cell sorting analysis showed that nearly all of the cells present were infected when a high MOI was used. In this sample, the size of which was limited, the cell lines derived from frugivorous African bats were significantly more susceptible than those derived from insectivorous American and Eurasian bats. Specifically, at most only ≈8% of the cells were infected in the case of the American Tb 1 Lu and B5L lines and the Eurasian MyDauLu/47.1 line when exposed to a high MOI of 5, whereas two African bat cell lines (R05T, EpoNi/22.1) were infected at rates approaching 100%. These results concur with those in recent reports of avian, swine, and human FLUAV infections in bat cell lines more limited in number than that tested in the present study (75, 76).

A single-cycle GFP reporter FLUAV was initially used to test a large number of conditions. We subsequently infected Tb 1 Lu cells with the replication-competent WSN strain to determine if this bat cell line can support the entire replication cycle of a human FLUAV. As Tb 1 Lu cells were relatively refractory to infection (Fig. 1), infection was initiated with an MOI of 0.1, which is relatively high for multicycle replication (Fig. 2). Viral titers increased over the course of the infection, exceeding 0.5×10^6 PFU/ml. In addition to the WSN lab strain, the primary human isolates

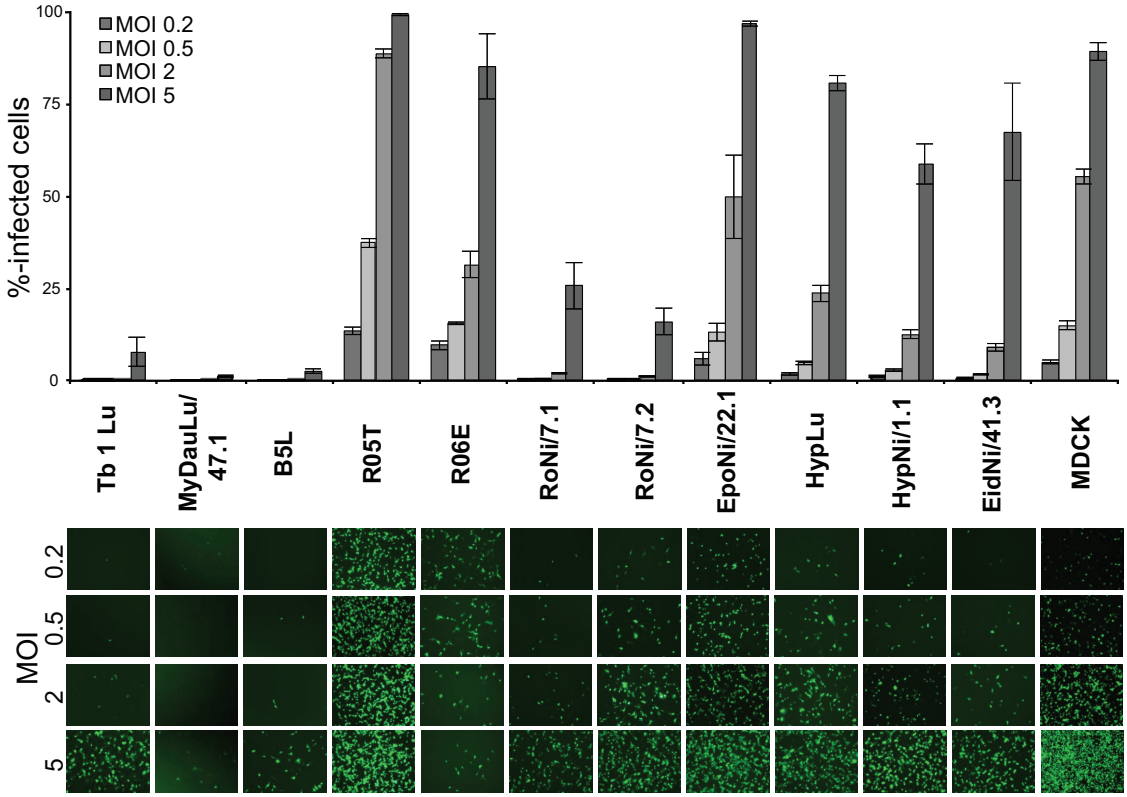

FIG 1 Cells from diverse bats are susceptible to infection with a human-origin influenza A virus (A/WSN/33). Bat cells were infected with virus at increasing MOIs of a single-cycle GFP reporter virus (A/WSN/33). After inoculation, the cells were imaged (bottom) and the percentage of infected cells was quantitated by fluorescence-activated cell sorting (top). Data represent means ± SDs ($n = 3$). Bat cells were derived from Brazilian free-tailed bats (Tb 1 Lu), eastern pipistrelles (B5L), Daubenton's myotis bats (MyDauLu/47.1), Egyptian rousettes (R05T, R06E, RoNi/7.1, RoNi/7.2), Büttikofer's epauletted fruit bats (EpoNi/22.1), hammer-headed fruit bats (HypLu/45.1, HypNi/1.1), and African straw-colored fruit bats (EidNi/41.3). Canine (MDCK) cells were included as a positive control.

A/Udorn/1972 (H3N2) and A/California/04/2009 (H1N1) and the influenza B virus (FLUBV) B/Brisbane/60/2008 also replicated in Tb 1 Lu cells (data not shown).

The FLUAV RdRP is a key determinant of host tropism and pathogenicity (36, 46, 61). We therefore tested the replicative capacity of viruses carrying RNP proteins from a primary human and avian FLUAV strains in Tb 1 Lu cells, A/New York/312/2001

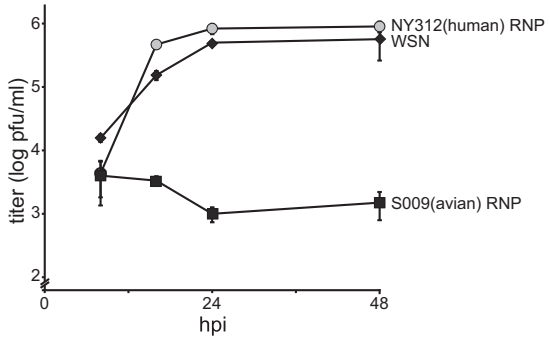

FIG 2 Restricted replication in bat cells of human influenza A virus (A/WSN/33) carrying an avian virus-derived RNP. The multicycle replication kinetics of WSN or reassortants carrying ribonucleoprotein genes from primary human (NY312) or avian (S009) isolates in Tb 1 Lu cells (MOI = 0.1) are shown. Data represent the mean titers ± SD ($n = 3$) determined by plaque assay.

(H1N1; NY312) and A/green-winged teal/OH/175/1986 (H2N1; S009), respectively (Fig. 2). The remaining genes were derived from the WSN strain to control for the variability that might be introduced by the differential receptor usage of human and avian viruses and focus on phenotypes primarily caused by the RNP genes. The PB2 subunit from the NY312 isolate carries the human signature K627, whereas that from the S009 isolate carries the avian signature E627. We and others have previously shown that replication of the S009 reassortant virus is selectively restricted in human cells but not avian or swine cells, consistent with the species-specific defect in polymerase activity attributed to PB2 E627 (55, 77). Virus carrying the NY312 RNP replicated similarly to WSN in Tb 1 Lu cells, reaching high titers within 24 h postinoculation (Fig. 2). Conversely, replication of the virus carrying the S009 RNP was dramatically impaired in these cells, with minimal replication being evidenced by a constant low-level viral titer. The titers of the S009 RNP virus were ≈3 log units lower than those of the WSN and NY312 RNP viruses. This contrasts with the highly efficient replication of the S009 RNP virus in avian cells where the polymerase and host are matched (55), indicating that this virus is not generically impaired. These data indicate that, akin to other mammalian cells (61), bat cells are a restrictive environment for the activity of an avian-origin FLUAV RdRP. Together, these data demonstrate that all of the host machinery required by human FLUAV to bind, enter, and express viral genes is present in diverse

bat cells and that Tb 1 Lu cells are also capable of at least producing infectious virions.

The bat FLUAV ribonucleoprotein complex possesses a limited reassortment capacity. Reassortment is frequently associated with an increasing host range and the emergence of pandemic viruses. Coinfection of bat cells with human and porcine H1N1 strains produced novel progeny virus reassortants, but it is not known if genes from bat FLUAV can reassort with other strains (75). A prerequisite for FLUAV genome reassortment is compatibility among the genes and gene products derived from different viruses. We therefore assessed the activity of reassortant FLUAV RdRPs containing components derived from human, avian, and bat viruses using a cell-based polymerase activity assay. The homotypic bat RNP containing subunits from A/bat/Guatemala/164/2009 (H17N10; Guat164) is highly active in human cells (Fig. 3A) (16, 17). Heterotypic RdRPs were created by individually exchanging the PB1, PB2, or PA subunit from the Guat164 RdRP with the PB1, PB2, or PA subunit from the human isolate WSN, NY312, A/Brevig Mission/1918 (H1N1; 1918), A/Udorn/1972 (H3N2; Udorn), or A/Utah/01/2009 (H1N1; UT1) RdRP or the avian isolate S009 RdRP (Fig. 3A). The enzymatic activity of all heterotypic RdRPs with human FLUAV subunits was reduced by a factor of at least 1,000. Only the avian S009 PB1 reassortant was marginally functional, retaining ≈5% the activity of the homotypic Guat164 RdRP.

We next tested whether human- and avian-derived FLUAV RdRPs could tolerate the introduction of individual bat-derived components. Polymerase activity was reconstituted with RNP subunits from WSN, NY312, 1918, UT1, Udorn, or S009, and heterotypic RdRPs were created by substituting components of the Guat164 RdRP (Fig. 3B). Enzymatic activity was normalized to that of the homotypic complex for each isolate, although the absolute activity of the avian S009 RdRP was severely restricted in human cells compared to that of the human RdRP, as previously published (55, 56). RdRP activity for most of the reassortants was appreciably compromised regardless of which RdRP subunit gene was exchanged; activity was reduced 20- to 1,000-fold compared to that of the homotypic RNPs. However, introducing Guat164 PA into the restricted avian S009 RdRP increased the activity by 4-fold.

PA reassortants can overcome the restriction of avian RdRPs in human cells (55, 56). We therefore further tested RdRP activity in avian cells to determine if the increased activity of the S009 reassortant containing the Guat164 PA subunit resulted from a generic enhancement of polymerase activity or a selective alleviation of avian polymerase restriction in human cells. Introducing bat PA into human and avian RdRPs reduced their activities in avian LMH cells (Fig. 3C). Western blotting confirmed that bat PA was expressed to equivalent levels in all RdRP activity assays. To exclude the possibility that the reassortants were defective because the Guat164 PA subunit does not function in avian cells, we confirmed that the homotypic bat RdRP and NP successfully reconstitute polymerase activity in avian cells (data not shown). Combined with the results from the RdRP activity assays in human cells, these data imply that bat PA partially alleviates the restriction of avian FLUAV RdRP in human cells. Thus, bat PA may increase the activity of an avian RdRP in a cell type-specific fashion, although most combinations of bat, human, and avian RdRP components impair activity, suggesting a limited capacity for reassortment between the known bat strains and other FLUAVs.

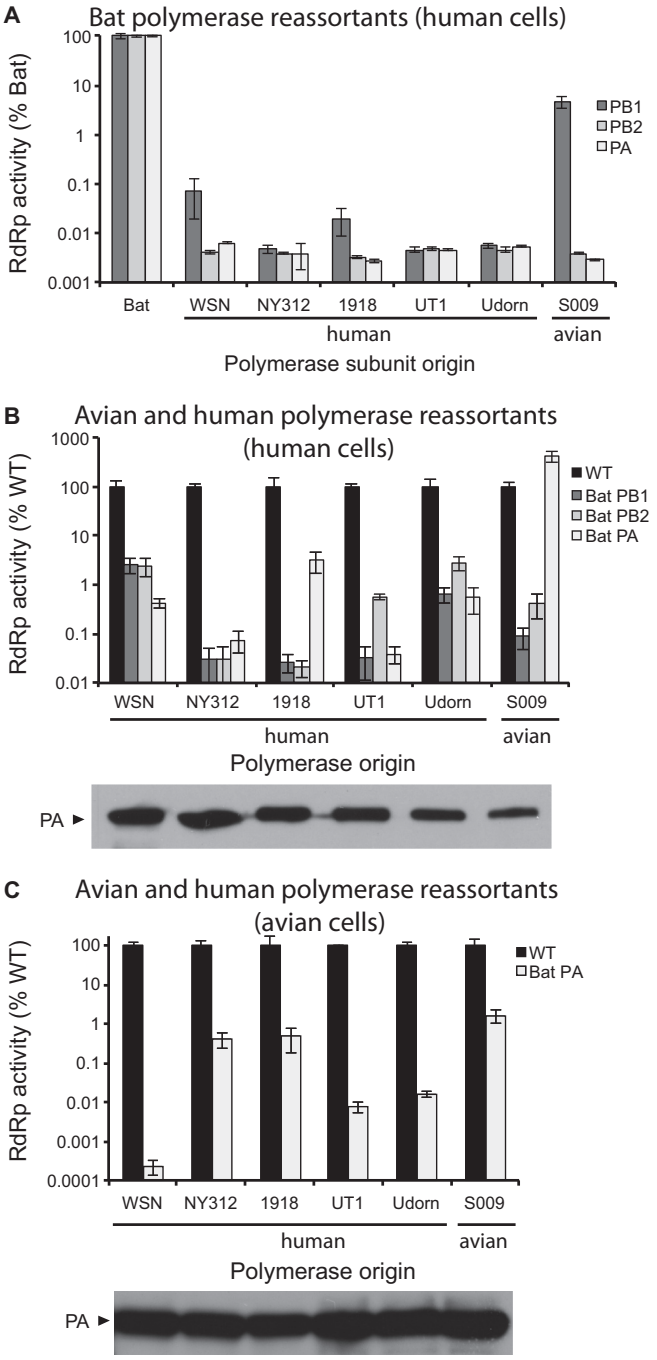

FIG 3 Limited capacity for reassortment with bat virus polymerase and nucleoprotein genes. (A) Polymerase activity was measured in HEK293T cells expressing bat FLUAV RdRP and NP. Individual subunits from the bat virus were replaced by those derived from human or avian FLUAV variants. Activity was normalized to that of the bat RdRP. (B) The activity of human- and avian-origin polymerase complexes or heterotypic polymerase complexes containing individual bat subunits was determined as described in the legend to panel A. Activity was normalized to that of the homotypic RdRP for each isolate and is expressed as a percentage of wild-type activity. The PA subunit was detected by Western blotting. (C) Activity of RdRPs containing a homotypic or bat PA subunit in avian LMH cells was determined as described in the legend to panel B. Data are reported as the mean ± SD ($n = 3$).

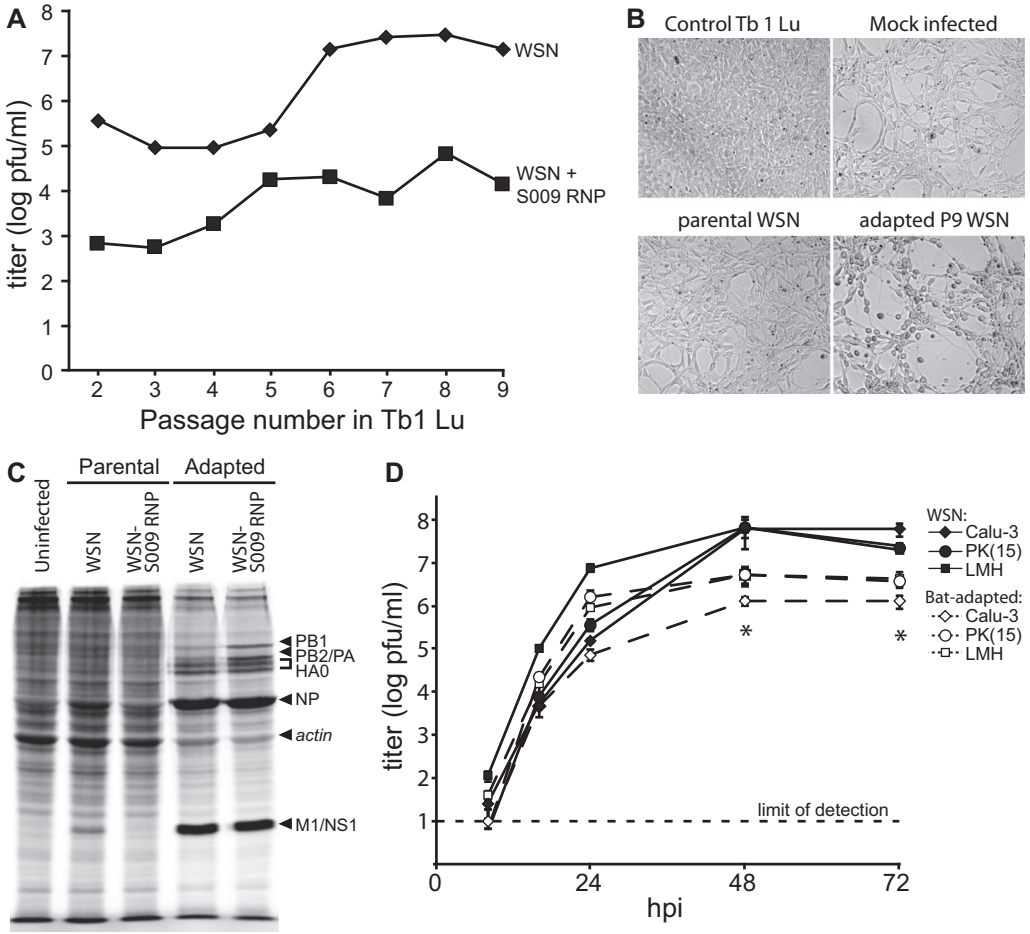

FIG 4 Experimental evolution of influenza A virus in bat cells increases virus replication and development of cytopathic effects. (A) Human-origin influenza A virus (A/WSN/33) or a reassortant virus carrying avian ribonucleoprotein genes (S009) was adapted to replication in Tb 1 Lu cells by serial blind passaging. At the indicated passages, viral titers were determined by plaque assay on MDCK cells. (B) Adapted virus induced severe cytopathic effects in Tb 1 Lu cells. Cells were infected at an MOI of 0.1 in virus growth medium and imaged at 15 h postinoculation. Control cells were left untreated. (C) Metabolic labeling of infected Tb 1 Lu cells reveals increased expression of viral proteins and enhanced host cell shutoff after inoculation with adapted viruses. Tb 1 Lu cells were infected at an MOI of 1, and proteins were radiolabeled at 9.5 h postinoculation. Lysates were separated by gel electrophoresis, and proteins were detected by phosphorimaging. Viral and host proteins are indicated. (D) Adapted viruses were attenuated in cells derived from nonbat hosts. Multicycle infections with parental WSN and the bat-adapted variant were analyzed in human (Calu-3), porcine [PK(15)], and avian (LMH) cells. Titers were determined at the indicated time points (hpi, hours postinfection) by plaque assay on MDCK cells. Data represent the mean ± SD ($n = 3$). Differences between parental and bat-adapted WSN viruses were statistically significant for all cell types beginning at 48 h postinoculation. *, $P < 0.05$.

Experimental adaptation of influenza A virus to replication in bat cells. To understand how FLUAV adapts to replication in unique cellular environments, we experimentally selected for efficient FLUAV replication in Tb1 Lu cells, a cell line that was relatively resistant to infection (Fig. 1). Two viruses, WSN and reassortant WSN carrying the avian S009 RNP, were evolved in parallel by blind passaging a 1:10 to 1:100 dilution of infected culture supernatant (MOI, ≈0.1) onto fresh Tb 1 Lu cells every 48 to 72 h for nine sequential rounds of infection (Fig. 4A). In agreement with our previous results (Fig. 2), WSN virus replicated at much higher levels than virus carrying the restricted S009 RNP. Titers for WSN remained relatively constant over 5 passages and then showed a marked 65-fold increase at passage 6. Subsequent passages yielded similarly high viral titers. Titers for WSN carrying the S009 RNP also increased throughout the experiment, beginning at passage 4. Despite increasing titers, this reassorted virus did not achieve the high levels detected for WSN virus. As the adaptation proceeded, the passaged virus began to induce cytopathic effects in the infected cells (Fig. 4B). In contrast, cells infected with the parental virus were indiscernible from mock-infected cells, with the observed cell retraction being due to the trypsin present in the virus growth medium (VGM) that was absent in the control cells. These results demonstrate that FLUAV evolves during serial passaging to replicate more efficiently and with greater cytopathogenicity in bat cells.

The increased replication and cytotoxicity of the adapted viruses could result from changes to any stage of the viral life cycle. To obtain a global overview of viral gene expression, Tb 1 Lu cells were metabolically labeled after inoculation with either the parental or the adapted virus (Fig. 4C). Protein expression levels in cells infected with the parental virus were marginally different from those in the uninfected control cells. Small amounts of NP and the comigrating M1 and NS1 proteins above the background amounts were discernible only in cells infected with WSN and not

TABLE 2 Analysis of SNPs of adapted FLUAV isolates[d]

Virus	Gene	Variant allele (nt)[a]	Mutation (aa)	Frequency (%)[b]			Bat allele (aa)[c]
				P0	P6	P9	
WSN	NS1	T251C	V84A	0	10.4	9.0	Q85
WSN	PA	T854A	M285K	0	65.6	77.6	M285
WSN	PA	A1187G	D396G	0	65.0	78.3	D/E391
WSN	PA	G1597A	E533K	0	5.7	0.0	E528
WSN	PB2	A1552G	I518V	0	28.5	31.7	V518
WSN + S009 RNP	NS1	T80C	L27P	0	0.0	7.9	E28
WSN + S009 RNP	NP	G394A	G132S	0	6.8	10.8	G131
WSN + S009 RNP	NP	A733G	S245G	0	29.7	41.1	G244
WSN + S009 RNP	PA	A1598C	E533A	0	0.0	16.5	E528
WSN + S009 RNP	PB2	G464A	S155N	0	0.0	27.6	S155
WSN + S009 RNP	PB2	G1879A	E627K	0	100.0	100.0	S627

[a] Variant nucleotide (nt) and nucleotide position within the open reading frame of the indicated gene.
[b] Percentage of all sequence reads containing the variant allele.
[c] Compared to A/little yellow-shouldered bat/Guatemala/164/2009 and A/flat-faced bat/Peru/033/2010 sequences.
[d] Viruses were deep sequenced at the indicated passage number (passage 0 [P0], P6, P9), and the sequences were compared to the reference genome sequences of the cDNA clones used during the initial rescue of the parental virus. Only variants detected at levels above the background are reported. aa, amino acid.

in cells with WSN carrying the S009 RNP, consistent with the restricted phenotype of the reassorted virus. In stark contrast, viral gene products were detected at high levels in cells infected with both of the adapted viruses (WSN and WSN-S009 RNP). Moreover, these viruses induced a pronounced decrease in the synthesis of host proteins compared to that for the uninfected control, indicative of the host shutoff associated with FLUAV infections (78). Adaptation resulted in viruses that exhibited a robust increase in viral protein synthesis while suppressing the expression of host proteins, potentially explaining the associated cytopathic effect.

Adaptation of the two FLUAVs to Tb 1 Lu cells may also include specialization to this cell type. To evaluate this possibility, cells from the three predominant hosts of FLUAV, human (Calu-3 cells), bird (LMH cells), and pig [PK(15) cells], were infected with the parental or bat-adapted version of WSN (Fig. 4D). Both parental and bat-adapted WSN replicated in all three cell types, whereas the adapted viruses reached peak viral titers 10- to 100-fold lower than those of the controls ($P < 0.05$ for all cell types beginning at 48 h postinoculation). Taken together, these results show that experimental evolution of FLUAV in bat cells results in virus with increased replication, cytopathogenicity, and viral gene expression in cells from the selection host at the cost of reduced replication in cells from the corresponding homotypic host.

Mutations in the viral polymerase mediate adaptation. FLUAVs isolated from bats are extremely divergent from all other sequenced strains (16, 17). Phylogenetic analysis places the bat FLUAV genes, with the exception of HA, as monophyletic outgroups to all other extant FLUAV genes. These genes diverged from those in the remaining FLUAV strains following the split from influenza B virus but before the diversification of FLUAV, indicating an ancient origin of the bat viruses. Furthermore, supporting a deep evolutionary divergence, the diversity found between select genes of the two bat FLUAV strains surpasses that found within all other FLUAV strains (17). The evolutionary distance between human and bat FLUAVs makes it challenging to predict which differences between these viruses, our adapted viruses, and their parental counterparts might contribute to differences in replication in bat cells. Therefore, the adapted and parental viruses were deep sequenced to identify the genetic basis underlying the adaptive phenotype. Input virus and samples from passages 6 and 9 were analyzed, and their sequences were compared to the sequences of the cDNA clones used during the initial rescue of the parental virus (Table 2). Sequences of parental stocks of both WSN and WSN carrying the S009 RNP were extremely homogeneous, lacking nucleotide diversity above a threshold of 1% (i.e., at each nucleotide position with over 100× coverage, less than 1% of all of the reads differed from the consensus residue), as would be expected for low-passage-number stocks that were rescued from cDNA clones.

As adaptation progressed, multiple nonsynonymous single nucleotide polymorphisms (SNPs) emerged in both viruses. SNPs were detected in PB2, PA, NP, and NS1 (Table 2). Most coded for amino acid residue changes that had not been previously associated with host adaptation (36). With the exception of the WSN PA G1597A SNP, these variants were retained and the majority increased in frequency by passage 9. Sequencing of the WSN-S009 RNP reassortant from passage 9 also revealed new SNPs in NS1, PA, and PB2.

None of the mutations present in the adapted viruses mapped to HA. This was unexpected, as HA frequently acquires changes to recognize species-specific variations in the sialic acid receptors used during viral entry (37–39). Moreover, the H17 and H18 proteins of FLUAV isolated from bats do not appear to use for virus entry the canonical sialic acids that are used by other influenza A viruses (17, 42, 45), yet the successful infections shown here were performed with the WSN isolate, which recognizes α2,6-sialosides and, to a much lesser extent, α2,3-sialosides (79). To determine if WSN uses sialic acid as a receptor for entry into bat cells, Tb 1 Lu cells were treated with receptor-destroying enzyme (RDE) or mock treated prior to infection with single-cycle GFP-WSN reporter virus. The neuraminidase activity in RDE nonspecifically removes sialic acid moieties. Although Tb 1 Lu cells were relatively resistant to infection with human FLUAV compared to the other bat cell lines, these cells were used as they are derived from the Brazilian free-tailed bat, whose geographic distribution overlaps the sites where both the H17N10 and H18N11 viruses were identified (Guatemala and Peru, respectively) (16, 17). Treatment with RDE prevented infection of Tb 1 Lu cells with the single-cycle

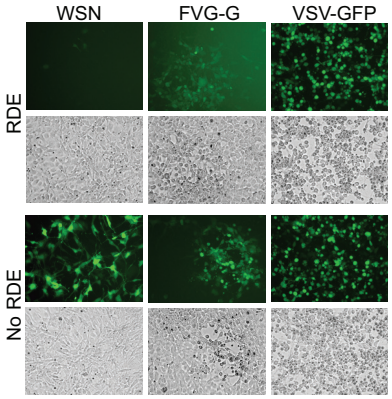

FIG 5 Sialic acids are receptors for entry of influenza A virus (A/WSN/33) into bat cells. Tb 1 Lu cells were treated with RDE or mock treated prior to infection with a single-cycle GFP reporter virus (WSN), a vesicular stomatitis virus G-pseudotyped influenza A virus carrying GFP (FVG-G), or VSV carrying GFP. Cells were imaged at 20 h postinoculation.

GFP-WSN reporter virus, whereas infection was unchanged in mock-treated cells (Fig. 5, No RDE). In parallel experiments, RDE- or mock-treated cells were infected with VSV-G-pseudotyped influenza virus carrying GFP (FVG-G) or VSV carrying GFP. RDE treatment had no effect on Tb 1 Lu cells infected with FVG-G or VSV, both of which enter cells independently of sialic acid receptors. Together, these results demonstrate that the canonical sialic acid receptors are present on bat cell lines and functional for entry of FLUAV. However, we cannot exclude the possibility that alternative receptors must be used to infect the appropriate target cell *in vivo* or that mutations in WSN HA would be necessary for transmission or infection in an animal.

Nine of the 11 mutations in the adapted viruses mapped to the viral replication machinery. Remarkably, the canonical PB2 E627K mutation associated with the adaptation of avian FLUAV RdRPs to mammalian hosts was detected in S009 PB2 and fixed completely by passage 6 (Table 2) (47). This polymorphism was absent from the parental stocks (i.e., it was present at a level below the level of detection). The G1879A mutation encoding E627K detected here differs from S009 mutations that we had generated for use in previous experiments, precluding the possibility of contamination as the source. The evolution of S009 PB2 K627 is consistent with the restrictive phenotype that we have characterized for Tb 1 Lu cells (Fig. 2) and parallels the emergence and complete fixation of PB2 K627 in only 6 days during mouse infections with an avian H5N1 isolate (80).

We constructed a series of mutant viruses to test the role of PB2 627 mutations during virus replication in Tb 1 Lu cells (Fig. 6A). To create a human-like RdRP, the S009 RdRP was mutated to carry both PB2 K627 and the G590S/Q591R (SR) polymorphism that contributed to the 2009 influenza pandemic (55, 69). Conversely, an avian virus-like polymerase was introduced into WSN by mutating PB2 to E627. These mutant viruses, along with their wild-type counterparts, were used to initiate infections in Tb 1 Lu cells. Viruses carrying RNPs from WSN and NY312 replicated to moderately high titers, whereas the virus carrying the S009 RNP was severely attenuated. The PB2 mutations completely converted the restriction phenotype. The humanized virus carrying S009 RNP replicated to levels similar to those of WSN and NY312 strains, whereas the avian virus-like WSN strain was restricted,

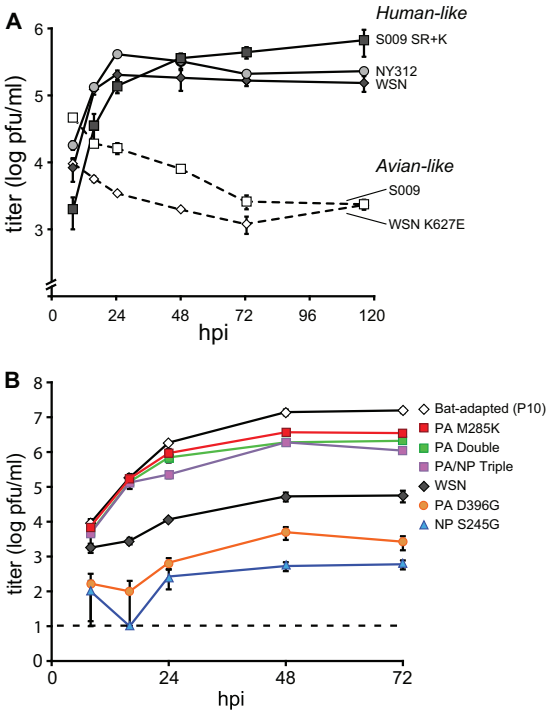

FIG 6 Adaptive mutations in the influenza A virus (A/WSN/33) RdRP regulate replication in bat cells. Deep sequencing of adapted viruses revealed high-frequency mutations in the PB2, PA, and NP genes that were then introduced into recombinant viruses. (A) The PB2 subunit from the avian H2N1 S009 isolate was humanized by insertion of the E627K and the SR mutations, whereas the WSN PB2 subunit was avianized by insertion of the K627E mutation. (B) The specified mutations were inserted alone or in combination into the parental WSN virus. The replication efficiency of all viruses (WSN, WSN mutants, bat-adapted virus) was assessed during multicycle replication experiments in Tb 1 Lu cells. Mean viral titers ± SD ($n = 3$) were determined by plaque assay on MDCK cells.

like the virus carrying the S009 RNP. Thus, mutations at PB2 position 627 control replication ability in bat cells, reinforcing the role of this region as a major regulator of FLUAV tropism in birds versus mammals.

Four other nonsynonymous polymorphisms were found at a high frequency in RNP subunits: NP S245G, PB2 I518V, PA M285K, and PA D396G (Table 2). The NP G245 and PB2 V518 mutations are present in all currently known bat FLUAV isolates. PB2 V518 predominates in other FLUAV isolates as well and was not pursued further. However, none of the PA mutations were present in bat FLUAV RdRP subunits and were exceedingly rare in subunits of other sequenced isolates (data not shown). The enrichment of NP S245G, PA M285K, and PA D396G during passaging could result from positive selection or represent hitchhiking. We therefore rescued recombinant FLUAV carrying these mutations alone or in combination and infected Tb 1 Lu cells. As in previous experiments, the parental WSN virus yielded titers almost 1,000-fold lower than those of the bat-adapted virus (Fig. 6B). Viruses carrying NP S245G or PA D396G were further impaired and replicated at levels at least another 10-fold lower than those for the parental WSN virus. In contrast, virus carrying only the PA M285K change replicated to high levels and had titers within 5-fold of those of the bat-adapted virus, almost completely phenocopying adaptation. Moreover, combining PA M285K with

the attenuating mutation NP S245G and/or PA D396G increased the replication of these viruses. These data identify PA K285 to be a potent enhancer of replication in Tb 1 Lu cells and a novel regulator of species-specific FLUAV replication.

DISCUSSION

The discovery of two ancient and diverse lineages of FLUAV in bats separated by >3,000 km over the course of 2 years implies the existence of a large, geographically dispersed cryptic reservoir for FLUAVs. As other viruses circulating in bats have spilled over into other mammals to cause disease outbreaks (20), it is of great interest to determine the potential for bats to be a source of FLUAV capable of emerging in the human population. We assessed the capacity of bats to serve as mixing vessels of FLUAVs capable of infecting humans by challenging a panel of bat cell lines with a prototypical mammalian FLUAV isolate. Every bat cell line tested, including those derived from Eurasian and African bats, was susceptible to FLUAV infection, implicating both New World and Old World bats as potential hosts. Moreover, FLUBV also replicated in bat cells. Experimental evolution of a FLUAV in bat cells rapidly gave rise to variants with a high replication capacity and cytopathogenicity. Bat cells exerted novel pressures, selecting for a new adaptive mutation in the PA subunit of human-origin virus, M285K, which solely conveyed enhanced replication in bat cells. In contrast to mutation, reassortment between bat, avian, and human viral polymerase genes failed to reconstitute highly active complexes or increase their host range. Thus, bat cells possess all of the necessary host machinery to support the robust replication of human influenza viruses. As the most recent common ancestor of humans and bats lived ≈65 million to 100 million years ago (81, 82), these findings suggest that human FLUAV and FLUBV exploit ancestral and well-conserved host factors during replication.

Experimental adaptation in bat cells showed that the viral RdRP was a hot spot; 7 of the 11 identified mutations accumulated in the PB2 and PA subunits. The adaptive M285K mutation in PA revealed by experimental evolution in bat cells is highly unusual and was detected only once in over 23,000 viruses isolated from humans or animals (data not shown), even though the polymerase complex is a frequent target of mutation as FLUAV changes hosts (36). The PA subunit has discrete N- and C-terminal domains and is essential for both transcription and replication of the viral genome. Like other FLUAV isolates, the N-terminal domain from the bat-origin H17N10 virus is an endonuclease important for viral transcription (83). The PA C-terminal domain containing the M285K change is best characterized as the PB1 binding site (84). Structures of this head-shaped domain reveal a "jaw" that encircles the extreme N terminus of PB1 (85, 86). Residue 285 is located outside the PB1 binding site, and the region surrounding this residue has not been assigned a discrete function. The N terminus of PB1 extends toward residue 285. The PA M285K change may enhance PB1 binding by interacting with the well-conserved aspartate at the second residue of PB1. PB1 D2 is important for high-affinity interactions with PA (87), yet the direct effect of PA M285K on these processes is unclear. PA M285K is part of a growing number of variations in PA that increase replication in a species-specific fashion, including variations carried by PA of avian H5N1 and H7N9 viruses that crossed over into humans (56, 58, 88–90).

The PB2 subunit has long been identified to be a host range determinant and a mediator of viral pathogenicity (48). The avian PB2 subunit was also under strong selective pressure in bat cells; the E627K mutation was rapidly fixed in the avian-origin polymerase and conveyed enhanced replication and cytopathic effects. The acquisition of the archetypal lysine at position 627 in PB2 during adaptation in bat cells suggests that the mode of restriction present in bats is similar to that present in other mammalian hosts. The bat-origin FLUAV does not carry either K627 or E627 in the PB2 subunit but carries the highly unusual S627. Bat-origin FLUAVs are the only viruses known to have this mutation, yet this polymerase is not restricted and functions at high levels in both human and avian cells (Fig. 3 and data not shown) (16, 17).

A potential contributor to the activity of bat polymerases in human cells is the presence of asparagine instead of the more common aspartate at residue 701 in bat-derived PB2 proteins. Residue D701 interacts with the bipartite nuclear localization signal of PB2 (91). The D701N substitution disrupts this interaction and has previously been shown to selectively enhance the nuclear import of PB2 in mammalian cells (92), replication in mice (59, 93), and transmission in guinea pigs (94). Neighboring PB2 residue 702 has also been implicated in host shifts. The amino acid present at position 702 was computationally identified as a signature residue indicative of avian (K702) or human (R702) viral origin (95–97). The mammalian signature residue R702 is absent from bat PB2 proteins with S702. Thus, studies of bat PB2 residues 701 and 702 will be important to determine their impact on polymerase function. Such studies will determine whether these residue changes alone are sufficient for the observed high-level activity of bat-origin polymerases in human cells or, perhaps, whether this viral lineage carries an alternative escape mechanism that avoids restriction.

To our surprise, none of the mutations that arose during experimental evolution were in HA or NA. HA and NA frequently change before or during cross-species transmission to recognize host-specific variations in the sialic acid receptors used by the virus for attachment to the cell surface (37–41). The HA of WSN, the virus used here, preferentially recognizes α2,6-sialosides (79). The stability of HA and NA during repeated replication cycles in bats suggests that the preferred receptor is present on these bat cells. This conclusion is supported by results showing that the enzymatic removal of sialic acid moieties from bat cells prevents infection by FLUAV but not viruses that utilize other entry pathways, including a pseudotyped FLUAV (Fig. 5). Neither HA nor NA from bat strains H17N10 and H18N11 recognizes α2,6-sialosides, α2,3-sialosides, or any of the over 600 other glycans tested (17, 42–44). FLUAVs use noncanonical pathways for sialic acid-independent entry, during which binding between cell surface C-type lectins, such as DC-SIGN and L-SIGN, and glycans on the virion occurs (98). Thus, H17 and H18 might recognize a highly unusual sialoside or utilize alternative receptors during infections with the bat isolates. Studies to determine whether similar receptor usage and patterns of adaptation occur during cross-species infection in intact organisms will be important, as in this setting FLUAV also has to evade mucosal and adaptive immunity to reach sufficient extracellular titers for further transmission.

The results presented here suggest that infections in evolutionarily diverse bats contribute to the broad host range and geographic distribution of FLUAV. Furthermore, the data demonstrate that bats represent a unique environment for FLUAV that rapidly enforces selective pressures on the viral polymerase for adaptive mutations that enhance viral replication. Thus, replica-

tion in nontraditional hosts like bats may be an important source of evolution and diversification of FLUAVs.

ACKNOWLEDGMENTS

We thank J. Taubenberger, Y. Kawaoka, P. Palese, D. Loeb, and R. Baric for sharing reagents, L. Moncla and T. Wesely for preliminary experiments, and B. Sugden and S. Radoshitzky for critical feedback. We are grateful to L. Bollinger (IRF—Frederick) for manuscript editing.

The content of this publication does not necessarily reflect the views or policies of the U.S. Department of Health and Human Services or of the institutions and companies affiliated with the authors.

Y.C. and J.H.K. performed this work as employees of Tunnell Government Services, Inc., and S.Y. performed this work as an employee of Battelle Memorial Institute. The work of these three authors was performed under Battelle's prime contract with NIAID (contract no. HHSN272200700016I).

This work was supported in part by the National Institute of General Medical Sciences (R00GM088484), a Shaw Scientist Award, a Mirus Research Award, and a Wisconsin Partnership Education and Research Committee New Investigator Program grant (grant 2563) to A.M.

REFERENCES

1. **Taubenberger JK, Kash JC.** 2010. Influenza virus evolution, host adaptation, and pandemic formation. Cell Host Microbe **7:**440–451. http://dx.doi.org/10.1016/j.chom.2010.05.009.
2. **Chambers TM, Dubovi EJ, Donis RO.** 2013. Equine/canine/feline/seal influenza, p 203–217. *In* Webster RG, Monto AS, Braciale TJ, Lamb RA (ed), Textbook of influenza, 2nd ed. John Wiley & Sons, Ltd, Oxford, United Kingdom.
3. **Neumann G, Noda T, Kawaoka Y.** 2009. Emergence and pandemic potential of swine-origin H1N1 influenza virus. Nature **459:**931–939. http://dx.doi.org/10.1038/nature08157.
4. **Wang TT, Palese P.** 2013. Emergence and evolution of the 1918, 1957, 1968, and 2009 pandemic virus strains, p 218–228. *In* Webster RG, Monto AS, Braciale TJ, Lamb RA (ed), Textbook of influenza, 2nd ed. John Wiley & Sons, Ltd, Oxford, United Kingdom.
5. **Webby R, Richt J.** 2013. Influenza in swine, p 190–202. *In* Webster RG, Monto AS, Braciale TJ, Lamb RA (ed), Textbook of influenza, 2nd ed. John Wiley & Sons, Ltd, Oxford, United Kingdom.
6. **Karlsson E, Engel G, Feeroz M, Sorn S, Rompis A, Lee B, Shaw E, Oh G, Schillaci M, Grant R, Heidrich J, Schultz-Cherry S, Jones-Engel L.** 2012. Evidence of influenza virus infection in nonhuman primates. Emerg. Infect. Dis. **18:**1672–1675. http://dx.doi.org/10.3201/eid1810.120214.
7. **Kuiken T, Rimmelzwaan G, van Riel D, van Amerongen G, Baars M, Fouchier R, Osterhaus A.** 2004. Avian H5N1 influenza in cats. Science **306:**241. http://dx.doi.org/10.1126/science.1102287.
8. **Songserm T, Amonsin A, Jam-on R, Sae-Heng N, Meemak N, Pariyothorn N, Payungporn S, Theamboonlers A, Poovorawan Y.** 2006. Avian influenza H5N1 in naturally infected domestic cat. Emerg. Infect. Dis. **12:**681–683. http://dx.doi.org/10.3201/eid1204.051396.
9. **Crawford PC, Dubovi EJ, Castleman WL, Stephenson I, Gibbs EP, Chen L, Smith C, Hill RC, Ferro P, Pompey J, Bright RA, Medina MJ, Johnson CM, Olsen CW, Cox NJ, Klimov AI, Katz JM, Donis RO.** 2005. Transmission of equine influenza virus to dogs. Science **310:**482–485. http://dx.doi.org/10.1126/science.1117950.
10. **Hurt AC, Vijaykrishna D, Butler J, Baas C, Maurer-Stroh S, Silva-de la-Fuente MC, Medina-Vogel G, Olsen B, Kelso A, Barr IG, González-Acuña D.** 2014. Detection of evolutionarily distinct avian influenza A viruses in Antarctica. mBio **5**(3):e01098-14. http://dx.doi.org/10.1128/mBio.01098-14.
11. **Hause BM, Collin EA, Liu R, Huang B, Sheng Z, Lu W, Wang D, Nelson EA, Li F.** 2014. Characterization of a novel influenza virus in cattle and swine: proposal for a new genus in the Orthomyxoviridae family. mBio **5**(2):e00031-14. http://dx.doi.org/10.1128/mBio.00031-14.
12. **Osterhaus AD, Rimmelzwaan GF, Martina BE, Bestebroer TM, Fouchier RA.** 2000. Influenza B virus in seals. Science **288:**1051–1053. http://dx.doi.org/10.1126/science.288.5468.1051.
13. **Geraci JR, St Aubin DJ, Barker IK, Webster RG, Hinshaw VS, Bean WJ, Ruhnke HL, Prescott JH, Early G, Baker AS, Madoff S, Schooley RT.** 1982. Mass mortality of harbor seals: pneumonia associated with influenza A virus. Science **215:**1129–1131. http://dx.doi.org/10.1126/science.7063847.
14. **Leyva-Grado VH, Mubareka S, Krammer F, Cardenas WB, Palese P.** 2012. Influenza virus infection in guinea pigs raised as livestock, Ecuador. Emerg. Infect. Dis. **18:**1135–1138. http://dx.doi.org/10.3201/eid1807.111930.
15. **Jackson D, Elderfield RA, Barclay WS.** 2011. Molecular studies of influenza B virus in the reverse genetics era. J. Gen. Virol. **92:**1–17. http://dx.doi.org/10.1099/vir.0.026187-0.
16. **Tong S, Li Y, Rivailler P, Conrardy C, Castillo DA, Chen LM, Recuenco S, Ellison JA, Davis CT, York IA, Turmelle AS, Moran D, Rogers S, Shi M, Tao Y, Weil MR, Tang K, Rowe LA, Sammons S, Xu X, Frace M, Lindblade KA, Cox NJ, Anderson LJ, Rupprecht CE, Donis RO.** 2012. A distinct lineage of influenza A virus from bats. Proc. Natl. Acad. Sci. U. S. A. **109:**4269–4274. http://dx.doi.org/10.1073/pnas.1116200109.
17. **Tong S, Zhu X, Li Y, Shi M, Zhang J, Bourgeois M, Yang H, Chen X, Recuenco S, Gomez J, Chen LM, Johnson A, Tao Y, Dreyfus C, Yu W, McBride R, Carney PJ, Gilbert AT, Chang J, Guo Z, Davis CT, Paulson JC, Stevens J, Rupprecht CE, Holmes EC, Wilson IA, Donis RO.** 2013. New World bats harbor diverse influenza A viruses. PLoS Pathog. **9:**e1003657. http://dx.doi.org/10.1371/journal.ppat.1003657.
18. **Mackenzie JS, Jeggo M.** 2013. Reservoirs and vectors of emerging viruses. Curr. Opin. Virol. **3:**170–179. http://dx.doi.org/10.1016/j.coviro.2013.02.002.
19. **Teeling EC, Springer MS, Madsen O, Bates P, O'Brien SJ, Murphy WJ.** 2005. A molecular phylogeny for bats illuminates biogeography and the fossil record. Science **307:**580–584. http://dx.doi.org/10.1126/science.1105113.
20. **Smith I, Wang LF.** 2013. Bats and their virome: an important source of emerging viruses capable of infecting humans. Curr. Opin. Virol. **3:**84–91. http://dx.doi.org/10.1016/j.coviro.2012.11.006.
21. **Calisher CH, Childs JE, Field HE, Holmes KV, Schountz T.** 2006. Bats: important reservoir hosts of emerging viruses. Clin. Microbiol. Rev. **19:**531–545. http://dx.doi.org/10.1128/CMR.00017-06.
22. **Towner JS, Amman BR, Sealy TK, Carroll SA, Comer JA, Kemp A, Swanepoel R, Paddock CD, Balinandi S, Khristova ML, Formenty PB, Albarino CG, Miller DM, Reed ZD, Kayiwa JT, Mills JN, Cannon DL, Greer PW, Byaruhanga E, Farnon EC, Atimnedi P, Okware S, Katongole-Mbidde E, Downing R, Tappero JW, Zaki SR, Ksiazek TG, Nichol ST, Rollin PE.** 2009. Isolation of genetically diverse Marburg viruses from Egyptian fruit bats. PLoS Pathog. **5:**e1000536. http://dx.doi.org/10.1371/journal.ppat.1000536.
23. **Towner JS, Pourrut X, Albarino CG, Nkogue CN, Bird BH, Grard G, Ksiazek TG, Gonzalez JP, Nichol ST, Leroy EM.** 2007. Marburg virus infection detected in a common African bat. PLoS One **2:**e764. http://dx.doi.org/10.1371/journal.pone.0000764.
24. **Halpin K, Young PL, Field HE, Mackenzie JS.** 2000. Isolation of Hendra virus from pteropid bats: a natural reservoir of Hendra virus. J. Gen. Virol. **81:**1927–1932. http://dx.doi.org/10.1099/vir.0.17031-0.
25. **Chua KB, Koh CL, Hooi PS, Wee KF, Khong JH, Chua BH, Chan YP, Lim ME, Lam SK.** 2002. Isolation of Nipah virus from Malaysian Island flying-foxes. Microbes Infect. **4:**145–151. http://dx.doi.org/10.1016/S1286-4579(01)01522-2.
26. **Li W, Shi Z, Yu M, Ren W, Smith C, Epstein JH, Wang H, Crameri G, Hu Z, Zhang H, Zhang J, McEachern J, Field H, Daszak P, Eaton BT, Zhang S, Wang LF.** 2005. Bats are natural reservoirs of SARS-like coronaviruses. Science **310:**676–679. http://dx.doi.org/10.1126/science.1118391.
27. **Lau SK, Woo PC, Li KS, Huang Y, Tsoi HW, Wong BH, Wong SS, Leung SY, Chan KH, Yuen KY.** 2005. Severe acute respiratory syndrome coronavirus-like virus in Chinese horseshoe bats. Proc. Natl. Acad. Sci. U. S. A. **102:**14040–14045. http://dx.doi.org/10.1073/pnas.0506735102.
28. **Annan A, Baldwin HJ, Corman VM, Klose SM, Owusu M, Nkrumah EE, Badu EK, Anti P, Agbenyega O, Meyer B, Oppong S, Sarkodie YA, Kalko EK, Lina PH, Godlevska EV, Reusken C, Seebens A, Gloza-Rausch F, Vallo P, Tschapka M, Drosten C, Drexler JF.** 2013. Human betacoronavirus 2c EMC/2012-related viruses in bats, Ghana and Europe. Emerg. Infect. Dis. **19:**456–459. http://dx.doi.org/10.3201/eid1903.121503.
29. **Ge XY, Li JL, Yang XL, Chmura AA, Zhu G, Epstein JH, Mazet JK, Hu B, Zhang W, Peng C, Zhang YJ, Luo CM, Tan B, Wang N, Zhu Y,**

Crameri G, Zhang SY, Wang LF, Daszak P, Shi ZL. 2013. Isolation and characterization of a bat SARS-like coronavirus that uses the ACE2 receptor. Nature **503:**535–538. http://dx.doi.org/10.1038/nature12711.

30. **Memish ZA, Mishra N, Olival KJ, Fagbo SF, Kapoor V, Epstein JH, Alhakeem R, Durosinloun A, Al Asmari M, Islam A, Kapoor A, Briese T, Daszak P, Al Rabeeah AA, Lipkin WI.** 2013. Middle East respiratory syndrome coronavirus in bats, Saudi Arabia. Emerg. Infect. Dis. **19:** 1819–1823. http://dx.doi.org/10.3201/eid1911.131172.
31. **Drexler JF, Corman VM, Muller MA, Maganga GD, Vallo P, Binger T, Gloza-Rausch F, Rasche A, Yordanov S, Seebens A, Oppong S, Adu Sarkodie Y, Pongombo C, Lukashev AN, Schmidt-Chanasit J, Stocker A, Carneiro AJ, Erbar S, Maisner A, Fronhoffs F, Buettner R, Kalko EK, Kruppa T, Franke CR, Kallies R, Yandoko ER, Herrler G, Reusken C, Hassanin A, Kruger DH, Matthee S, Ulrich RG, Leroy EM, Drosten C.** 2012. Bats host major mammalian paramyxoviruses. Nat. Commun. **3:**796. http://dx.doi.org/10.1038/ncomms1796.
32. **Quan PL, Firth C, Conte JM, Williams SH, Zambrana-Torrelio CM, Anthony SJ, Ellison JA, Gilbert AT, Kuzmin IV, Niezgoda M, Osinubi MO, Recuenco S, Markotter W, Breiman RF, Kalemba L, Malekani J, Lindblade KA, Rostal MK, Ojeda-Flores R, Suzan G, Davis LB, Blau DM, Ogunkoya AB, Alvarez Castillo DA, Moran D, Ngam S, Akaibe D, Agwanda B, Briese T, Epstein JH, Daszak P, Rupprecht CE, Holmes EC, Lipkin WI.** 2013. Bats are a major natural reservoir for hepaciviruses and pegiviruses. Proc. Natl. Acad. Sci. U. S. A. **110:**8194–8199. http://dx.doi.org/10.1073/pnas.1303037110.
33. **Drexler JF, Geipel A, Konig A, Corman VM, van Riel D, Leijten LM, Bremer CM, Rasche A, Cottontail VM, Maganga GD, Schlegel M, Muller MA, Adam A, Klose SM, Carneiro AJ, Stocker A, Franke CR, Gloza-Rausch F, Geyer J, Annan A, Adu-Sarkodie Y, Oppong S, Binger T, Vallo P, Tschapka M, Ulrich RG, Gerlich WH, Leroy E, Kuiken T, Glebe D, Drosten C.** 2013. Bats carry pathogenic hepadnaviruses antigenically related to hepatitis B virus and capable of infecting human hepatocytes. Proc. Natl. Acad. Sci. U. S. A. **110:**16151–16156. http://dx.doi.org/10.1073/pnas.1308049110.
34. **Luis AD, Hayman DT, O'Shea TJ, Cryan PM, Gilbert AT, Pulliam JR, Mills JN, Timonin ME, Willis CK, Cunningham AA, Fooks AR, Rupprecht CE, Wood JL, Webb CT.** 2013. A comparison of bats and rodents as reservoirs of zoonotic viruses: are bats special? Proc. Biol. Sci. **280:**20122753. http://dx.doi.org/10.1098/rspb.2012.2753.
35. **Neumann G, Kawaoka Y.** 2006. Host range restriction and pathogenicity in the context of influenza pandemic. Emerg. Infect. Dis. **12:**881–886. http://dx.doi.org/10.3201/eid1206.051336.
36. **Manz B, Schwemmle M, Brunotte L.** 2013. Adaptation of avian influenza A virus polymerase in mammals to overcome the host species barrier. J. Virol. **87:**7200–7209. http://dx.doi.org/10.1128/JVI.00980-13.
37. **Rogers GN, Paulson JC, Daniels RS, Skehel JJ, Wilson IA, Wiley DC.** 1983. Single amino acid substitutions in influenza haemagglutinin change receptor binding specificity. Nature **304:**76–78. http://dx.doi.org/10.1038/304076a0.
38. **Herfst S, Schrauwen EJ, Linster M, Chutinimitkul S, de Wit E, Munster VJ, Sorrell EM, Bestebroer TM, Burke DF, Smith DJ, Rimmelzwaan GF, Osterhaus AD, Fouchier RA.** 2012. Airborne transmission of influenza A/H5N1 virus between ferrets. Science **336:**1534–1541. http://dx.doi.org/10.1126/science.1213362.
39. **Imai M, Watanabe T, Hatta M, Das SC, Ozawa M, Shinya K, Zhong G, Hanson A, Katsura H, Watanabe S, Li C, Kawakami E, Yamada S, Kiso M, Suzuki Y, Maher EA, Neumann G, Kawaoka Y.** 2012. Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets. Nature **486:** 420–428.
40. **Baum LG, Paulson JC.** 1991. The N2 neuraminidase of human influenza virus has acquired a substrate specificity complementary to the hemagglutinin receptor specificity. Virology **180:**10–15. http://dx.doi.org/10.1016/0042-6822(91)90003-T.
41. **Kobasa D, Kodihalli S, Luo M, Castrucci MR, Donatelli I, Suzuki Y, Suzuki T, Kawaoka Y.** 1999. Amino acid residues contributing to the substrate specificity of the influenza A virus neuraminidase. J. Virol. **73:**6743–6751.
42. **Sun X, Shi Y, Lu X, He J, Gao F, Yan J, Qi J, Gao GF.** 2013. Bat-derived influenza hemagglutinin H17 does not bind canonical avian or human receptors and most likely uses a unique entry mechanism. Cell Rep. **3:**769–778. http://dx.doi.org/10.1016/j.celrep.2013.01.025.
43. **Li Q, Sun X, Li Z, Liu Y, Vavricka CJ, Qi J, Gao GF.** 2012. Structural and functional characterization of neuraminidase-like molecule N10 derived from bat influenza A virus. Proc. Natl. Acad. Sci. U. S. A. **109:** 18897–18902. http://dx.doi.org/10.1073/pnas.1211037109.
44. **Zhu X, Yang H, Guo Z, Yu W, Carney PJ, Li Y, Chen LM, Paulson JC, Donis RO, Tong S, Stevens J, Wilson IA.** 2012. Crystal structures of two subtype N10 neuraminidase-like proteins from bat influenza A viruses reveal a diverged putative active site. Proc. Natl. Acad. Sci. U. S. A. **109:** 18903–18908. http://dx.doi.org/10.1073/pnas.1212579109.
45. **Zhu X, Yu W, McBride R, Li Y, Chen LM, Donis RO, Tong S, Paulson JC, Wilson IA.** 2013. Hemagglutinin homologue from H17N10 bat influenza virus exhibits divergent receptor-binding and pH-dependent fusion activities. Proc. Natl. Acad. Sci. U. S. A. **110:**1458–1463. http://dx.doi.org/10.1073/pnas.1218509110.
46. **Mehle A, McCullers JA.** 2013. Structure and function of the influenza virus replication machinery and PB1-F2, p 133–145. *In* Webster RG, Monto AS, Braciale TJ, Lamb RA (ed), Textbook of influenza, 2nd ed. John Wiley & Sons, Ltd, Oxford, United Kingdom.
47. **Subbarao EK, London W, Murphy BR.** 1993. A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. J. Virol. **67:**1761–1764.
48. **Almond JW.** 1977. A single gene determines the host range of influenza virus. Nature **270:**617–618. http://dx.doi.org/10.1038/270617a0.
49. **Mehle A, Doudna JA.** 2008. An inhibitory activity in human cells restricts the function of an avian-like influenza virus polymerase. Cell Host Microbe **4:**111–122. http://dx.doi.org/10.1016/j.chom.2008.06.007.
50. **Labadie K, Dos Santos Afonso E, Rameix-Welti MA, van der Werf S, Naffakh N.** 2007. Host-range determinants on the PB2 protein of influenza A viruses control the interaction between the viral polymerase and nucleoprotein in human cells. Virology **362:**271–282. http://dx.doi.org/10.1016/j.virol.2006.12.027.
51. **Cauldwell AV, Moncorge O, Barclay WS.** 2013. Unstable polymerase-nucleoprotein interaction is not responsible for avian influenza virus polymerase restriction in human cells. J. Virol. **87:**1278–1284. http://dx.doi.org/10.1128/JVI.02597-12.
52. **Paterson D, Te Velthuis AJ, Vreede FT, Fodor E.** 2014. Host restriction of influenza virus polymerase activity by PB2 627E is diminished on short viral templates in a nucleoprotein-independent manner. J. Virol. **88:** 339–344. http://dx.doi.org/10.1128/JVI.02022-13.
53. **Hatta M, Gao P, Halfmann P, Kawaoka Y.** 2001. Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. Science **293:** 1840–1842. http://dx.doi.org/10.1126/science.1062882.
54. **Salomon R, Franks J, Govorkova EA, Ilyushina NA, Yen HL, Hulse-Post DJ, Humberd J, Trichet M, Rehg JE, Webby RJ, Webster RG, Hoffmann E.** 2006. The polymerase complex genes contribute to the high virulence of the human H5N1 influenza virus isolate A/Vietnam/1203/04. J. Exp. Med. **203:**689–697. http://dx.doi.org/10.1084/jem.20051938.
55. **Mehle A, Doudna JA.** 2009. Adaptive strategies of the influenza virus polymerase for replication in humans. Proc. Natl. Acad. Sci. U. S. A. **106:**21312–21316. http://dx.doi.org/10.1073/pnas.0911915106.
56. **Mehle A, Dugan VG, Taubenberger JK, Doudna JA.** 2012. Reassortment and mutation of the avian influenza virus polymerase PA subunit overcome species barriers. J. Virol. **86:**1750–1757. http://dx.doi.org/10.1128/JVI.06203-11.
57. **Bussey KA, Bousse TL, Desmet EA, Kim B, Takimoto T.** 2010. PB2 residue 271 plays a key role in enhanced polymerase activity of influenza A viruses in mammalian host cells. J. Virol. **84:**4395–4406. http://dx.doi.org/10.1128/JVI.02642-09.
58. **Bussey KA, Desmet EA, Mattiacio JL, Hamilton A, Bradel-Tretheway B, Bussey HE, Kim B, Dewhurst S, Takimoto T.** 2011. PA residues in the 2009 H1N1 pandemic influenza virus enhance avian influenza virus polymerase activity in mammalian cells. J. Virol. **85:**7020–7028. http://dx.doi.org/10.1128/JVI.00522-11.
59. **Gabriel G, Dauber B, Wolff T, Planz O, Klenk HD, Stech J.** 2005. The viral polymerase mediates adaptation of an avian influenza virus to a mammalian host. Proc. Natl. Acad. Sci. U. S. A. **102:**18590–18595. http://dx.doi.org/10.1073/pnas.0507415102.
60. **Garten RJ, Davis CT, Russell CA, Shu B, Lindstrom S, Balish A, Sessions WM, Xu X, Skepner E, Deyde V, Okomo-Adhiambo M, Gubareva L, Barnes J, Smith CB, Emery SL, Hillman MJ, Rivailler P, Smagala J, de Graaf M, Burke DF, Fouchier RA, Pappas C, Alpuche-Aranda CM, Lopez-Gatell H, Olivera H, Lopez I, Myers CA, Faix D, Blair PJ, Yu C, Keene KM, Dotson PD, Jr, Boxrud D, Sambol AR, Abid**

SH, St George K, Bannerman T, Moore AL, Stringer DJ, Blevins P, Demmler-Harrison GJ, Ginsberg M, Kriner P, Waterman S, Smole S, Guevara HF, Belongia EA, Clark PA, Beatrice ST, et al. 2009. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. Science **325:**197–201. http://dx.doi.org/10.1126/science.1176225.

61. **Naffakh N, Tomoiu A, Rameix-Welti MA, van der Werf S.** 2008. Host restriction of avian influenza viruses at the level of the ribonucleoproteins. Annu. Rev. Microbiol. **62:**403–424. http://dx.doi.org/10.1146/annurev.micro.62.081307.162746.
62. **Smith GJ, Vijaykrishna D, Bahl J, Lycett SJ, Worobey M, Pybus OG, Ma SK, Cheung CL, Raghwani J, Bhatt S, Peiris JS, Guan Y, Rambaut A.** 2009. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature **459:**1122–1125. http://dx.doi.org/10.1038/nature08182.
63. **Howden KJ, Brockhoff EJ, Caya FD, McLeod LJ, Lavoie M, Ing JD, Bystrom JM, Alexandersen S, Pasick JM, Berhane Y, Morrison ME, Keenliside JM, Laurendeau S, Rohonczy EB.** 2009. An investigation into human pandemic influenza virus (H1N1) 2009 on an Alberta swine farm. Can. Vet. J. **50:**1153–1161.
64. **Marsh GA, Hatami R, Palese P.** 2007. Specific residues of the influenza A virus hemagglutinin viral RNA are important for efficient packaging into budding virions. J. Virol. **81:**9727–9736. http://dx.doi.org/10.1128/JVI.01144-07.
65. **Taubenberger JK, Reid AH, Lourens RM, Wang R, Jin G, Fanning TG.** 2005. Characterization of the 1918 influenza virus polymerase genes. Nature **437:**889–893. http://dx.doi.org/10.1038/nature04230.
66. **Regan JF, Liang Y, Parslow TG.** 2006. Defective assembly of influenza A virus due to a mutation in the polymerase subunit PA. J. Virol. **80:**252–261. http://dx.doi.org/10.1128/JVI.80.1.252-261.2006.
67. **Neumann G, Fujii K, Kino Y, Kawaoka Y.** 2005. An improved reverse genetics system for influenza A virus generation and its implications for vaccine production. Proc. Natl. Acad. Sci. U. S. A. **102:**16825–16829. http://dx.doi.org/10.1073/pnas.0505587102.
68. **Hoffmann E, Neumann G, Kawaoka Y, Hobom G, Webster RG.** 2000. A DNA transfection system for generation of influenza A virus from eight plasmids. Proc. Natl. Acad. Sci. U. S. A. **97:**6108–6113. http://dx.doi.org/10.1073/pnas.100133697.
69. **Yamada S, Hatta M, Staker BL, Watanabe S, Imai M, Shinya K, Sakai-Tagawa Y, Ito M, Ozawa M, Watanabe T, Sakabe S, Li C, Kim JH, Myler PJ, Phan I, Raymond A, Smith E, Stacy R, Nidom CA, Lank SM, Wiseman RW, Bimber BN, O'Connor DH, Neumann G, Stewart LJ, Kawaoka Y.** 2010. Biological and structural characterization of a host-adapting amino acid in influenza virus. PLoS Pathog. **6:**e1001034. http://dx.doi.org/10.1371/journal.ppat.1001034.
70. **Matrosovich M, Matrosovich T, Garten W, Klenk HD.** 2006. New low-viscosity overlay medium for viral plaque assays. Virol. J. **3:**63. http://dx.doi.org/10.1186/1743-422X-3-63.
71. **Schott DH, Cureton DK, Whelan SP, Hunter CP.** 2005. An antiviral role for the RNA interference machinery in Caenorhabditis elegans. Proc. Natl. Acad. Sci. U. S. A. **102:**18420–18424. http://dx.doi.org/10.1073/pnas.0507123102.
72. **Hao L, Sakurai A, Watanabe T, Sorensen E, Nidom CA, Newton MA, Ahlquist P, Kawaoka Y.** 2008. Drosophila RNAi screen identifies host genes important for influenza virus replication. Nature **454:**890–893. http://dx.doi.org/10.1038/nature07151.
73. **Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR.** 2001. Universal primer set for the full-length amplification of all influenza A viruses. Arch. Virol. **146:**2275–2289. http://dx.doi.org/10.1007/s007050170002.
74. **Biesold SE, Ritz D, Gloza-Rausch F, Wollny R, Drexler JF, Corman VM, Kalko EK, Oppong S, Drosten C, Muller MA.** 2011. Type I interferon reaction to viral infection in interferon-competent, immortalized cell lines from the African fruit bat Eidolon helvum. PLoS One **6:**e28131. http://dx.doi.org/10.1371/journal.pone.0028131.
75. **Dlugolenski D, Jones L, Tompkins SM, Crameri G, Wang LF, Tripp RA.** 2013. Bat cells from Pteropus alecto are susceptible to influenza A virus infection and reassortment. Influenza Other Respir. Viruses **7:**900–903. http://dx.doi.org/10.1111/irv.12128.
76. **Hoffmann M, Muller MA, Drexler JF, Glende J, Erdt M, Gutzkow T, Losemann C, Binger T, Deng H, Schwegmann-Wessels C, Esser KH, Drosten C, Herrler G.** 2013. Differential sensitivity of bat cells to infection by enveloped RNA viruses: coronaviruses, paramyxoviruses, filoviruses, and influenza viruses. PLoS One **8:**e72942. http://dx.doi.org/10.1371/journal.pone.0072942.
77. **Jagger BW, Memoli MJ, Sheng ZM, Qi L, Hrabal RJ, Allen GL, Dugan VG, Wang R, Digard P, Kash JC, Taubenberger JK.** 2010. The PB2-E627K mutation attenuates viruses containing the 2009 H1N1 influenza pandemic polymerase. mBio **1**(1):e00067-10. http://dx.doi.org/10.1128/mBio.00067-10.
78. **Gale M, Jr, Tan SL, Katze MG.** 2000. Translational control of viral gene expression in eukaryotes. Microbiol. Mol. Biol. Rev. **64:**239–280. http://dx.doi.org/10.1128/MMBR.64.2.239-280.2000.
79. **Leung HS, Li OT, Chan RW, Chan MC, Nicholls JM, Poon LL.** 2012. Entry of influenza A virus with a alpha2,6-linked sialic acid binding preference requires host fibronectin. J. Virol. **86:**10704–10713. http://dx.doi.org/10.1128/JVI.01166-12.
80. **Min JY, Santos C, Fitch A, Twaddle A, Toyoda Y, DePasse JV, Ghedin E, Subbarao K.** 2013. Mammalian adaptation in the PB2 gene of avian H5N1 influenza virus. J. Virol. **87:**10884–10888. http://dx.doi.org/10.1128/JVI.01016-13.
81. **O'Leary MA, Bloch JI, Flynn JJ, Gaudin TJ, Giallombardo A, Giannini NP, Goldberg SL, Kraatz BP, Luo ZX, Meng J, Ni X, Novacek MJ, Perini FA, Randall ZS, Rougier GW, Sargis EJ, Silcox MT, Simmons NB, Spaulding M, Velazco PM, Weksler M, Wible JR, Cirranello AL.** 2013. The placental mammal ancestor and the post-K-Pg radiation of placentals. Science **339:**662–667. http://dx.doi.org/10.1126/science.1229237.
82. **Zhang G, Cowled C, Shi Z, Huang Z, Bishop-Lilly KA, Fang X, Wynne JW, Xiong Z, Baker ML, Zhao W, Tachedjian M, Zhu Y, Zhou P, Jiang X, Ng J, Yang L, Wu L, Xiao J, Feng Y, Chen Y, Sun X, Zhang Y, Marsh GA, Crameri G, Broder CC, Frey KG, Wang LF, Wang J.** 2013. Comparative analysis of bat genomes provides insight into the evolution of flight and immunity. Science **339:**456–460. http://dx.doi.org/10.1126/science.1230835.
83. **Tefsen B, Lu G, Zhu Y, Haywood J, Zhao L, Deng T, Qi J, Gao GF.** 2014. The N-terminal domain of PA from bat-derived influenza-like virus H17N10 has endonuclease activity. J. Virol. **88:**1935–1941. http://dx.doi.org/10.1128/JVI.03270-13.
84. **Perez DR, Donis RO.** 2001. Functional analysis of PA binding by influenza A virus PB1: effects on polymerase activity and viral infectivity. J. Virol. **75:**8127–8136. http://dx.doi.org/10.1128/JVI.75.17.8127-8136.2001.
85. **He X, Zhou J, Bartlam M, Zhang R, Ma J, Lou Z, Li X, Li J, Joachimiak A, Zeng Z, Ge R, Rao Z, Liu Y.** 2008. Crystal structure of the polymerase PA(C)-PB1(N) complex from an avian influenza H5N1 virus. Nature **454:**1123–1126. http://dx.doi.org/10.1038/nature07120.
86. **Obayashi E, Yoshida H, Kawai F, Shibayama N, Kawaguchi A, Nagata K, Tame JR, Park SY.** 2008. The structural basis for an essential subunit interaction in influenza virus RNA polymerase. Nature **454:**1127–1131. http://dx.doi.org/10.1038/nature07225.
87. **Wunderlich K, Juozapaitis M, Ranadheera C, Kessler U, Martin A, Eisel J, Beutling U, Frank R, Schwemmle M.** 2011. Identification of high-affinity PB1-derived peptides with enhanced affinity to the PA protein of influenza A virus polymerase. Antimicrob. Agents Chemother. **55:**696–702. http://dx.doi.org/10.1128/AAC.01419-10.
88. **Yamayoshi S, Yamada S, Fukuyama S, Murakami S, Zhao D, Uraki R, Watanabe T, Tomita Y, Macken C, Neumann G, Kawaoka Y.** 2014. Virulence-affecting amino acid changes in the PA protein of H7N9 influenza A viruses. J. Virol. **88:**3127–3134. http://dx.doi.org/10.1128/JVI.03155-13.
89. **Manz B, Brunotte L, Reuther P, Schwemmle M.** 2012. Adaptive mutations in NEP compensate for defective H5N1 RNA replication in cultured human cells. Nat. Commun. **3:**802. http://dx.doi.org/10.1038/ncomms1804.
90. **Song J, Feng H, Xu J, Zhao D, Shi J, Li Y, Deng G, Jiang Y, Li X, Zhu P, Guan Y, Bu Z, Kawaoka Y, Chen H.** 2011. The PA protein directly contributes to the virulence of H5N1 avian influenza viruses in domestic ducks. J. Virol. **85:**2180–2188. http://dx.doi.org/10.1128/JVI.01975-10.
91. **Tarendeau F, Boudet J, Guilligay D, Mas PJ, Bougault CM, Boulo S, Baudin F, Ruigrok RW, Daigle N, Ellenberg J, Cusack S, Simorre JP, Hart DJ.** 2007. Structure and nuclear import function of the C-terminal domain of influenza virus polymerase PB2 subunit. Nat. Struct. Mol. Biol. **14:**229–233. http://dx.doi.org/10.1038/nsmb1212.
92. **Gabriel G, Herwig A, Klenk HD.** 2008. Interaction of polymerase subunit PB2 and NP with importin alpha1 is a determinant of host range

of influenza A virus. PLoS Pathog. **4:**e11. http://dx.doi.org/10.1371/journal.ppat.0040011.

93. **Li Z, Chen H, Jiao P, Deng G, Tian G, Li Y, Hoffmann E, Webster RG, Matsuoka Y, Yu K.** 2005. Molecular basis of replication of duck H5N1 influenza viruses in a mammalian mouse model. J. Virol. **79:**12058–12064. http://dx.doi.org/10.1128/JVI.79.18.12058-12064.2005.
94. **Steel J, Lowen AC, Mubareka S, Palese P.** 2009. Transmission of influenza virus in a mammalian host is increased by PB2 amino acids 627K or 627E/701N. PLoS Pathog. **5:**e1000252. http://dx.doi.org/10.1371/journal.ppat.1000252.
95. **Finkelstein DB, Mukatira S, Mehta PK, Obenauer JC, Su X, Webster RG, Naeve CW.** 2007. Persistent host markers in pandemic and H5N1 influenza viruses. J. Virol. **81:**10292–10299. http://dx.doi.org/10.1128/JVI.00921-07.
96. **Miotto O, Heiny AT, Albrecht R, Garcia-Sastre A, Tan TW, August JT, Brusic V.** 2010. Complete-proteome mapping of human influenza A adaptive mutations: implications for human transmissibility of zoonotic strains. PLoS One **5:**e9025. http://dx.doi.org/10.1371/journal.pone.0009025.
97. **Tamuri AU, Dos Reis M, Hay AJ, Goldstein RA.** 2009. Identifying changes in selective constraints: host shifts in influenza. PLoS Comput. Biol. **5:**e1000564. http://dx.doi.org/10.1371/journal.pcbi.1000564.
98. **Londrigan SL, Turville SG, Tate MD, Deng YM, Brooks AG, Reading PC.** 2011. N-linked glycosylation facilitates sialic acid-independent attachment and entry of influenza A viruses into cells expressing DC-SIGN or L-SIGN. J. Virol. **85:**2990–3000. http://dx.doi.org/10.1128/JVI.01705-10.
99. **Kühl A, Hoffmann M, Müller MA, Munster VJ, Gnirß K, Kiene M, Tsegaye TS, Behrens G, Herrler G, Feldmann H, Drosten C, Pöhlmann S.** 2011. Comparative analysis of Ebola virus glycoprotein interactions with human and bat cells. J. Infect. Dis. **204:**S840–S849. http://dx.doi.org/10.1093/infdis/jir306.
100. **Huynh J, Li S, Yount B, Smith A, Sturges L, Olsen JC, Nagel J, Johnson JB, Agnihothram S, Gates JE, Frieman MB, Baric RS, Donaldson EF.** 2012. Evidence supporting a zoonotic origin of human coronavirus strain NL63. J. Virol. **86:**12816–12825. http://dx.doi.org/10.1128/JVI.00906-12.
101. **Jordan I, Horn D, Oehmke S, Leendertz FH, Sandig V.** 2009. Cell lines from the Egyptian fruit bat are permissive for modified vaccinia Ankara. Virus Res. **145:**54–62. http://dx.doi.org/10.1016/j.virusres.2009.06.007.